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Principal Investigator:

Dr.Ms.Paramjit Grover, Senior Principal Scientist, Biology Division,

Indian Institute of Chemical Technology, Hyderabad, India

Tel: +91-40-27193135, E.mail:paramgrover@gmail.com, grover@iict.res.in

Co-Investigators:

Dr.M.F.Rahman, Senior Technical Officer,

Indian Institute of Chemical Technology, Hyderabad, India

Dr.M.Mahboob, Senior Technical Officer,

Indian Institute of Chemical Technology, Hyderabad, India

Project Co-coordinator:

Dr.USN Murty, Chief Scientist and Head Biology Division,

Indian Institute of Chemical Technology, Hyderabad, India

Tel: +91-40-27193134,E.mail:murty_usn@yahoo.com

AFOSR/AOARD Program Manager: Lt Col Thomas Erstfeld

Collaborators: Dr. Saber Hussain, Senior Scientist, AFRL, WPAFB, OH

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14. ABSTRACT
A study on the physicochemical properties and MTT cytotoxicity assay of cerium oxide (CeO2), cobalt oxide (Co3O4) and tungsten oxide (WO3) nanomaterials (NMs) on different cell lines was carried out during the last year. The physicochemical properties like size, size distribution, state of dispersion and zeta potential of these NMs are the important factors to study their toxicity.

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Abstract

A study on the physicochemical properties and MTT cytotoxicity assay of cerium oxide (CeO_2), cobalt oxide (Co_3O_4) and tungsten oxide (WO_3) nanomaterials (NMs) on different cell lines was carried out during the last one year. The physicochemical properties like size, size distribution, state of dispersion and zeta potential of these NMs are the important factors to study their toxicity. We developed a novel *in vitro* system to systematically assess the interaction of the NMs and their bulk within the cell. Bulk analogues were used to find out the size effect on the toxicity. The MTT cytotoxicity assay was performed in the four cell lines viz. human hepatocarcinoma cell line (Hep G2), human adinocarcinoma cell line (A549), human embryonic kidney cell line (HEK 293), human neuroblastoma cell line (IMR 32). The mean size of CeO_2, Co_3O_4, and WO_3 NMs was 25, 16 and 52 nm respectively. The DLS data revealed the aggregation of CeO_2, Co_3O_4, and WO_3 NMs in suspension. Zeta potential of CeO_2, Co_3O_4, and WO_3 NMs in DMEM was determined by LDV and found to be -7.74, -8.20, and -6.03 respectively. In the culture medium NMs showed a slight increase in the size with a concomitant decrease in zeta-potential. The MTT cytotoxicity assay with four cell lines exhibited dose dependent loss in the viability with CeO_2, Co_3O_4, and WO_3 NMs. There were variations in the sensitivity of cells to the different NMs. The cell lines exposed to CeO_2-NM for 24 hours exhibited IC_{50} <300 µg/ml except IMR 32 (>300 µg/ml). Similarly, among the cell lines exposed to Co_3O_4-NM only IMR32 and Hep G2 showed IC_{50} <300 µg/ml whereas WO_3-NM exposure did not show IC_{50} <300 µg/ml in any cell lines. Bulk compounds of these three metal oxides were less cytotoxic than their counter part NMs with all the four cell lines tested and the IC_{50} observed was > 300 µg/ml.

Introduction

Nanotechnologies are the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale level. It is a rapidly advancing discipline with a wide range of applications, including those in medicine and industry. Nanoparticles have a specific capacity for drug loading, high superparamagnetism, efficient photoluminescence, in the targeted delivery of imaging agents and anti-cancer drugs. Potential targets include organs such as the brain, which are normally protected by specialized barriers (such as the blood–brain barrier). Nanomaterials (NMs) can be defined as materials which have

at least one dimension less than 100nm. NMs exhibit very different properties from their bulk materials due to their unique physico-chemical features such as small size and large surface area, high mechanical, thermal and electrical strengths, increased solubility in water as well as their amenability for chemical manipulation. The overwhelming applications of NMs due to their superior physicochemical features bestow enormous potential for human exposure and environmental release. Therefore, using NMs without fully understanding potential health risks would be dangerous (Nel et al., 2006). The current lack of knowledge in this regard has led to an urgent call for the establishment of principles and test procedures to ensure the safe manufacture and use of NMs in the marketplace. Little is known about the clinical risks of exposure or whether NMs exposure may pose a risk to a fetus during pregnancy. Inhalation of nanoparticles or nanotubes is thought to be a risk for cardio respiratory disease. Although the placenta, lung, gastrointestinal tract and skin have been cited as barriers to many NMs, there is some, albeit conflicting, evidence those NMs from external exposures could translocate to other systemic sites. In the current investigation, the toxicological interaction of cerium oxide (CeO_2), cobalt oxide (Co_3O_4) and tungsten oxide (WO_3) NMs with various cell lines was studied.

CeO_2-NMs are used in solar cells, gas sensors & oxygen. They have been proved to be useful for treating glaucoma & catalyze reaction for cleaner fuel for future. Co_3O_4 NMs applications are in information storage, magnetic fluid, catalysts, pigments, coatings, catalysis, sensors, anode materials in rechargeable batteries, solar energy absorbers etc. WO_3 NMs are of great interest due to their potential use as electrochromic, gas sensing and photo catalyst materials. Therefore, there is urgent requirement for the toxicity study of these NMs at the cellular level in order to understand and conclude the real site of action of these NMs.

This research is intended to get the finer details regarding the physicochemical properties of NMs and to develop a novel *in vitro* system to systematically assess the NMs interaction within cells.

Experiment

1. Chemicals:

Cerium oxide bulk (CeO_2 , 99.9%, <5µm, CAS No.1306-38-3) ,Cerium oxide nanopowder (CeO_2 <25nm, CAS No.1306-38-3),,Cobalt oxide nanopowder (Co_3O_4 99.8%, <50nm CAS No. 138-06-1), Cobalt oxide bulk (Co_3O_4 99.8%, <10µm CAS No. 138-06-1), Tungsten oxide nanopowder (WO_3 99.8%, <100nm CAS No. 1314-35-8), Tungsten oxide bulk (WO_3 99%, <20µm CAS No. 1314-35-8) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Phosphate buffered saline (Ca_2+, Mg_2+ free; PBS), Dulbecco's modified eagle medium (DMEM), trypsin–EDTA, fetal bovine serum (FBS), antibiotic solution (10,000 U/ml penicillin,10 mg/ml streptomycin) were also purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals were obtained locally and were of analytical reagent grade. Cell culture plastic wares were obtained from Tarsons Products Pvt.Ltd. (Kolkata, India).

Cell culture:

- The human adinocarcinoma cell line (A549) was grown in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate, 2mM L-Glutamine, 1% Na-Pyruvate and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.
- Human hepatocarcinoma cell line (Hep G2) was grown in DMEM supplemented with 20% FBS, 0.2% sodium bicarbonate, 1% non essential amino acids, 2mM L-Glutamine and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.
- Human embryonic kidney cell line (HEK 293) was grown in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.
- Human neuroblastoma cell line (IMR 32) was obtained from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.

2. Characterization

Transmission Electron Microscopy:

Transmission Electron Microscope (TEM) characterization was performed to obtain nanoparticles size and morphology on a TEM (JEOL, Japan) at an accelerating voltage of 120 kV. CeO_2, Co_3O_4 and WO_3 nanoparticles were examined after suspension in Milli-Q water and subsequent deposition onto TEM grids. Information on mean size and standard error was calculated by measuring over 100 nanoparticles in random fields of view, in addition to images that show general morphology of the nanoparticles.

Dynamic light scattering (DLS) and Laser Doppler velocimetry (LDV):

Dynamic light scattering (DLS) and zeta potential measurements were performed with a Zetasizer Nano ZS (Malvern Instruments), provided with a He/Ne laser of 633 nm wavelength. DLS and Laser Doppler velocimetry (LDV) were used for the size and charge characterization of CeO_2, Co_3O_4 and WO_3 nanoparticles in solution, after suspension in DMEM with ultrasonication. The freshly prepared stock solutions were ultrasonicated using a probe sonicator for 10 min. Samples thus prepared were transferred to a 1.5 ml square cuvette for DLS measurements and 1 ml was transferred to a Malvern Clear Zeta Potential cell for LDV measurement. Average size was calculated by the software from the intensity, volume and number distributions measured.

Dispersion of test materials

Metal oxide nanoparticles (Ceo_2, Co_3O_4 and WO_3) and their bulk were dispersed in PBS. Homogenous dispersion was obtained by physical mixing and sonication for 5-10 minutes. Different stock solutions of metal oxide nanoparticles were obtained by diluting with DMEM with 5% FBS to obtain finale concentration of 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300µg/ml. 10ul of nanoparticles suspension was added to 100ul of exposure media in 96 well plate. Constant mixing was done before exposure to prevent the settle down of nanoparticles in the solution.

3. Cell viability (*MTT Cytotoxicity Assay)*

Metal oxide nanoparticles (CeO_2, Co_3O_4 and WO_3) cytotoxicity was assessed using MTT assay following the method described by Hansen et al., (1989) for this A549, Hep G2, HEK 293 and IMR 32 were used.

The assay is dependent on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product dissolved in DMSO and read at 570 nm. Briefly 100µl of mammalian cell lines were suspended in 96 wells plate after 50-60% confluency cells were treated to different concentrations of nanomaterials suspended in DMEM media with 5% serum for a time period of 24hr. Then, medium in each well was discarded and fresh supplemented medium (100 µl) followed by 10 µl of MTT solution (5 mg/ml in PBS, filtered sterile) was added. Medium blank was put up with only medium (100 µl) and MTT (10 µl). Plates were incubated at 37 °C for 2 h. The formazan crystals formed by the action of mitochondrial dehydrogenase on MTT was dissolved in 100ul of DMSO, Absorbance was measured at 570 nm using Spectra Max plus 384 UV-Visible plate reader.

Statistical analysis:

The statistical significant change in MTT assay between treated and control groups were analyzed by one-way ANOVA. Results were expressed as mean ± standard deviation (S.D.). Multiple comparisons were performed by Dunnett test. All calculations were performed using Graph Pad Prism 4 Software package for windows. The statistical significance for all tests was set at $p<0.05$.

Results and Discussion

Characterization of CeO_2, Co_3O_4 and WO_3 nanoparticles:

TEM was used to characterize size and morphology of CeO_2, Co_3O_4 and WO_3 nanoparticles. Mean size was calculated by measuring over 100 nanoparticles in random field. The mean of CeO_2 nanoparticle was 25 (Fig.1), for Co_3O_4 nanoparticle was 16 (Fig.2) and for WO_3 nanoparticle was 52 nm (Fig.3), respectively. The DLS data revealed the aggregation of CeO_2,

Co_3O_4 and WO_3 nanoparticles in suspension, which could be possibly due to physico-chemical interactions between the nanoparticles. Hence, in order to create homogenous solution, constant resuspension is necessary prior to use. Zeta potential (ζ) of CeO_2, Co_3O_4 and WO_3 NPs in DMEM was determined by LDV, and found to be -7.74, -8.20 and -6.03 respectively. The size and charge of CeO_2, Co_3O_4 and WO_3 NPs in DMEM using TEM, DLS and LDV respectively are presented in Table 1.

Our results on cell viability MTT assay with four cell lines i.e. IMR 32, A549, Hep G2 and HEK 293 have exhibited dose dependent loss in viability with CeO_2, Co_3O_4 and WO_3 NMs (Figures 13 to 24).

CeO_2-NMs significantly inhibited cell viability with IMR 32 cell lines at concentrations 40 - 300 µg/ml. Further, CeO_2-Bulk also significantly inhibited cell viability but from 200 - 300 µg/ml only (Fig.13). Similarly, CeO_2-NMs significantly inhibited percent viability in A549, Hep G2 and HEK 293 cell lines from 30 - 300 µg/ml concentrations. However, CeO_2-Bulk was not significant at all the concentrations tested with A549 and Hep G2 cell lines (Fig. 14& 15) but it significantly inhibited viability from concentrations 150 to 300 µg/ml (Fig. 16). In support to our result a study by Rosenkranz et al., 2012 revealed that CeO_2 NMs were more toxic than the micro CeO_2 as CeO_2 NMs significantly reduced the mitochondrial metabolism and hence cell viability by MTT assay in H4IIE rat hepatoma cell line when compared to the control.

Co_3O_4-NMs significantly inhibited cell viability in IMR 32 cell lines at the concentration range of 30 - 300 µg/ml, whereas Co_3O_4-Bulk significantly inhibited cell viability from 150 - 300 µg/ml concentrations (Fig.17). Similarly, Co_3O_4-NMs showed significant inhibition in A549 cell lines from 50 - 300 µg/ml concentrations, whereas Co_3O_4-Bulk was significant only at 300 µg/ml concentration (Fig. 18). In Hep G2 cell lines cell viability was significantly inhibited by Co_3O_4-NMs from 40 - 300 µg/ml concentrations, whereas Co_3O_4-Bulk had significant inhibition from 150 to 300 µg/ml concentrations (Fig. 19). Similarly, in HEK 293 cell lines Co_3O_4-NMs significantly inhibited cell viability from concentration range of 150 - 300 µg/ml, whereas CO_3O_4-Bulk inhibited cell viability significantly from 200 - 300 µg/ml concentrations (Fig. 20).In various studies Co_3O_4 nanoparticles were found cytotoxic and genotoxic. **Papis et al., 2009** demonstrated that the engineered Co_3O_4NMs readily entered the cell and caused loss in

cell viability when studied with Hep G2 and ECV-302 cell lines. Similarly, Colognato et al., 2008 showed the genotoxicity of cobalt nanoparticles in human peripheral leukocytes *in vitro*.

In IMR 32 cell lines WO_3-NMs significantly inhibited cell viability from 50 - 300 µg/ml concentrations, whereas WO_3-Bulk was not significant at any of concentrations tested (Fig. 21). Similarly, in A549 cell lines WO_3-NMs revealed significant inhibition at 300 µg/ml, whereas WO_3-Bulk was not at all significant at any concentration tested (Fig. 22). However, WO_3-NMs significantly inhibited viability of cells from 40 - 300 µg/ml in Hep G2 cell lines, whereas in HEK 293 cell lines the inhibitions were from 50 - 300 µg/ml concentrations. But, WO_3-Bulk was significantly inhibited from 150 - 300 µg/ml concentrations in Hep G2 cell lines, whereas in HEK 293 cell lines it was significant at 250 and 300 µg/ml concentrations only (Fig. 23 & 24). Studies on the toxicology of tungsten oxide NMs are not available.

The IC_{50} observed for the Co_3O_4-NMs with IMR 32 cell lines was 248.94 µg/ml, whereas other two NMs CeO_2 and WO_3 showed IC_{50} > 300 µg/ml. In case of A549 cell lines CeO_2-NMs exhibited IC_{50} 180.80 µg/ml, whereas with Co_3O_4-NMs and WO_3-NMs the values were >300 µg/ml. Similarly, with Hep G2 cell lines the IC_{50} observed was 202.47 and 132.37 with CeO_2-NMs and Co_3O_4-NMs respectively, whereas WO_3-NMs showed values which were >300 µg/ml. Further, with HEK 293 cell lines CeO_2-NMs exhibited IC_{50} of 180.91, whereas other two NMs i.e. Co_3O_4 and WO_3 the IC_{50} values were > 300 µg/ml (Table 2). These results have suggested that with all the four cell lines CeO_2-NMs were more potently cytotoxic followed by Co_3O_4-NMs. However, WO_3-NMs were least cytotoxic and the IC_{50} observed was >300 µg/ml with all the four cell lines. However, Bulk compounds of these three metal oxides were less cytotoxic than their counter part NMs with all the four cell lines tested and the IC_{50} observed was > 300 µg/ml (Table 2).

The further studies to be done in the future comprises,

- ✓ To evaluate the cell response to NMs following exposure using oxidative stress, apoptosis and gene expression and to correlate to NP characterization versus their interaction with cells.

- ✓ To understand cellular uptake mechanisms:
  - Do NMs enter the cells?
  - Which cellular organelles do they localize/located?

- Do they remain in NMs form in cells?
- What quantity of NM is taken up by the cells?

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1. Nel A, Xis T, Madler L, Li N. 2006. Toxic potential of materials at the nano-level. *Science* **311**: 622-627.
2. Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a preciseand rapid dye method for measuring cell growth / cell kill. *J. Immunol. Methods* **119**: 203-210.
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4. Papis E, Rossi F, Raspanti M, Dalle-Donne I, Colombo G, Milzani A, Bernardini G, Gornati R. 2009. Engineered cobalt oxide nanoparticles readily enter cells. *Toxicol. letters* **189**: 253-259.
5. Colognato R, Binelli A, Ponti J, Farina M, Bergamaschi E, Sabbioni E, Migliore L. 2008. Comparative genotoxicity of cobalt nanoparticles and ions on human peripheral leukocytes *in vitro*. *Mutagenesis* **23**: 377-382.

Table: 1. The size and charge of CeO_2, Co_3O_4 and WO_3 nanoparticles in DMEM.

Nano-material	Size using TEM (nm)	DLS		LDV		
		Average diameter (nm)	PDI	Zeta potential ζ (mV)	Electrophoretic Mobility (μm·cm/V/s)	pH
CeO_2	25	269.7	0.436	-7.74	-1.25	7.4
Co_3O_4	16	195.6	0.345	-8.20	-0.75	7.4
WO_3	52	203.0	0.411	-6.03	-0.75	7.4

Nanomaterials were dispersed in DMEM medium, mixing was done via probe sonication for 10min PDI=polydispersity index, DLS= dynamic light scattering, LDV= laser Doppler velocimetry, DMEM= Dulbecco's Modified Eagle's Medium

Fig. 1 - TEM image of CeO_2 nanoparticles

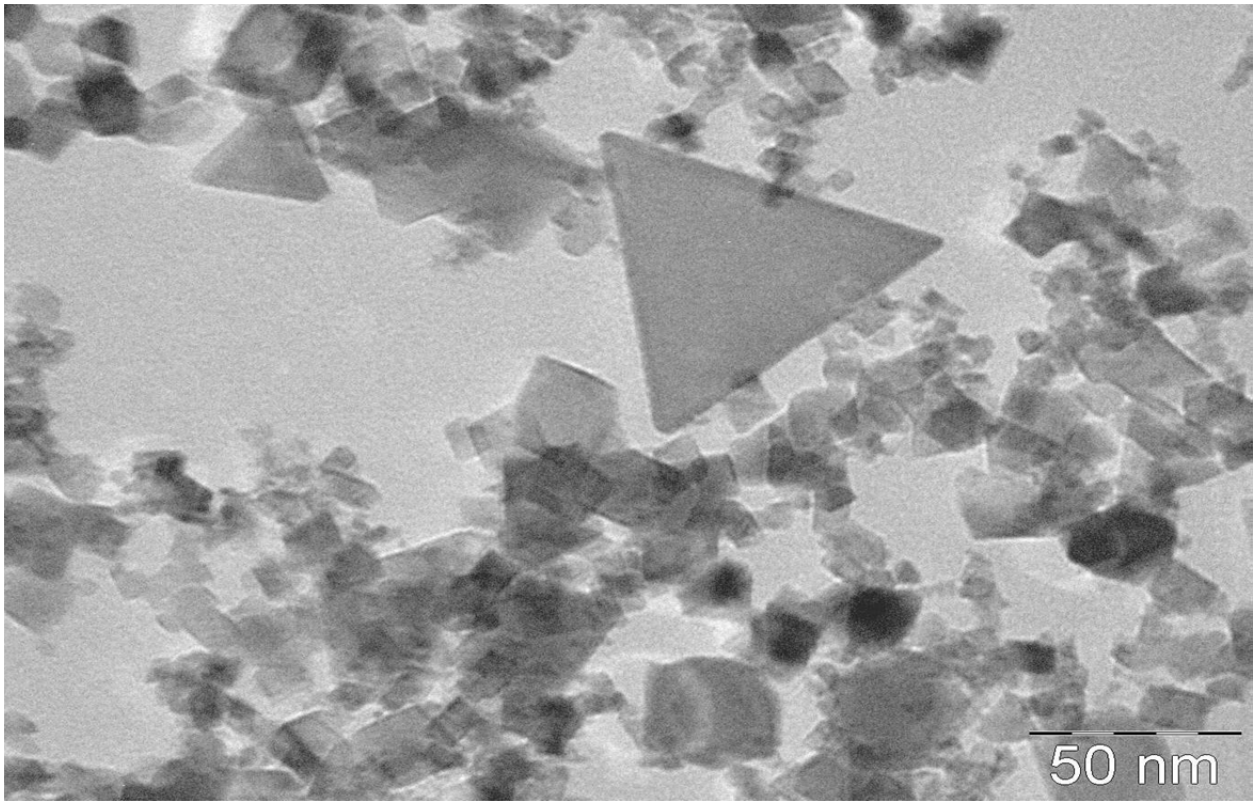


Fig. 2 - TEM image of Co_3O_4 nanoparticles

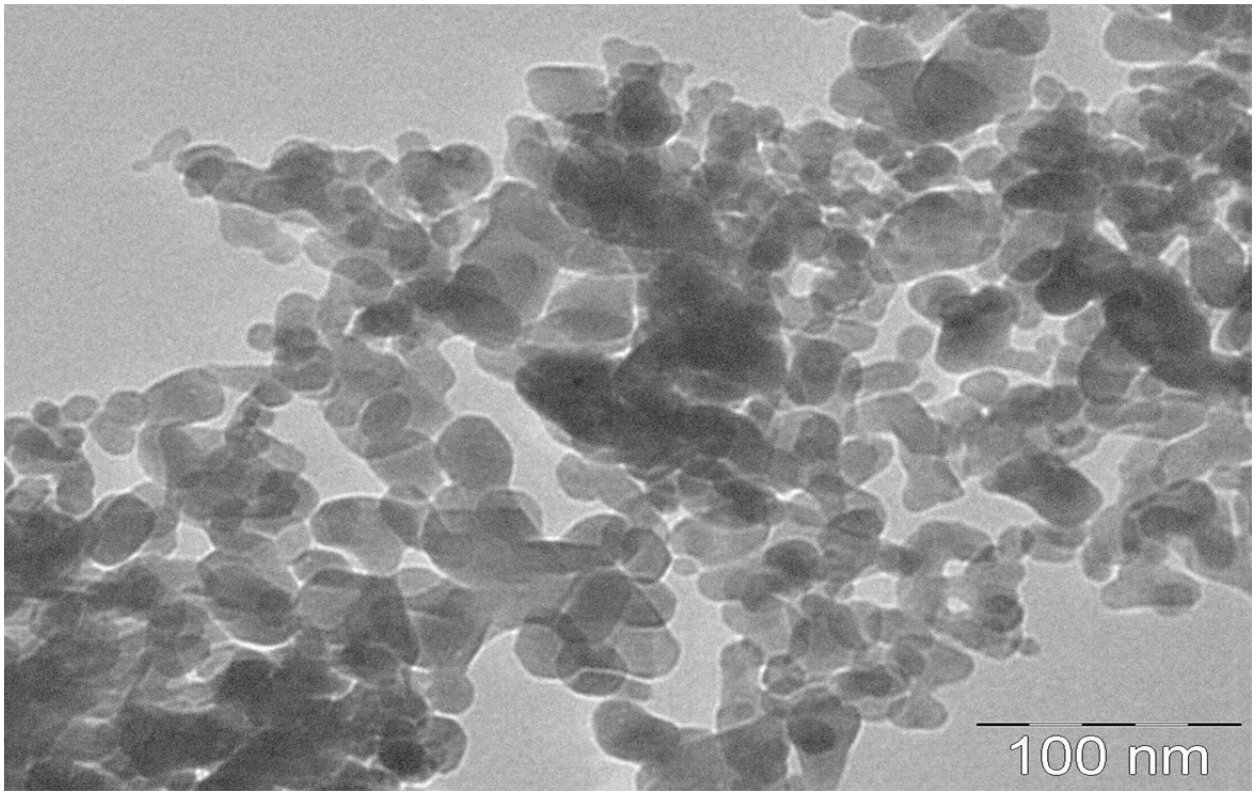


Fig. 3 - TEM image of WO_3 nanoparticles

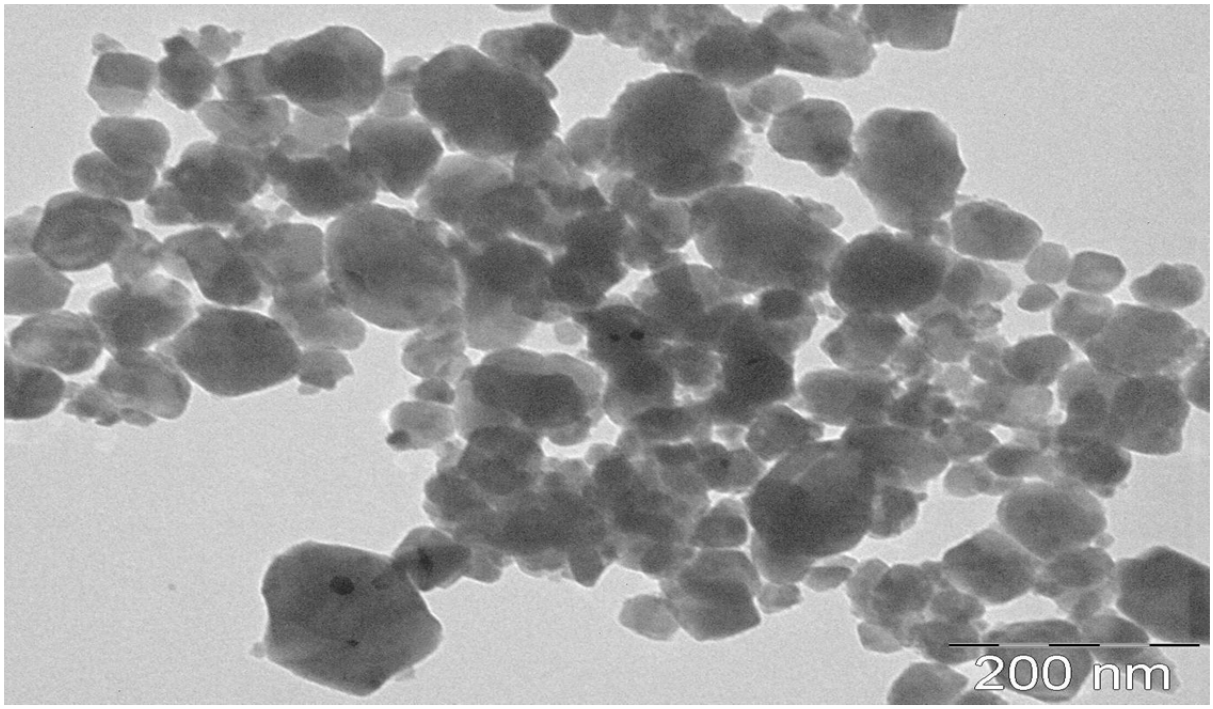


Fig. 4 - Size distribution of CeO_2 nanoparticles in DMEM

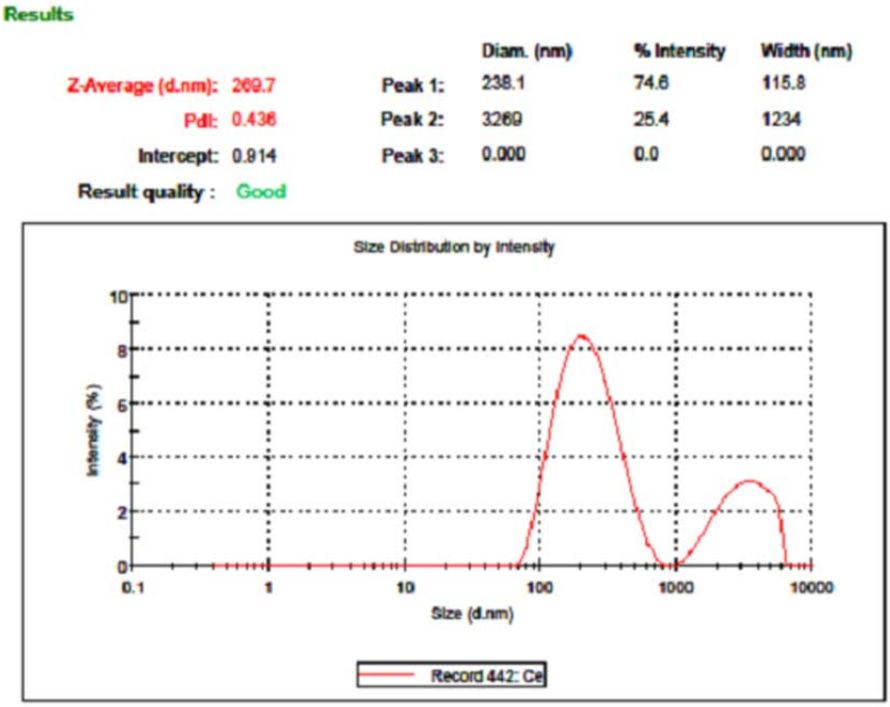


Fig. 5 - Zeta potential of CeO2 nanoparticles in DMEM

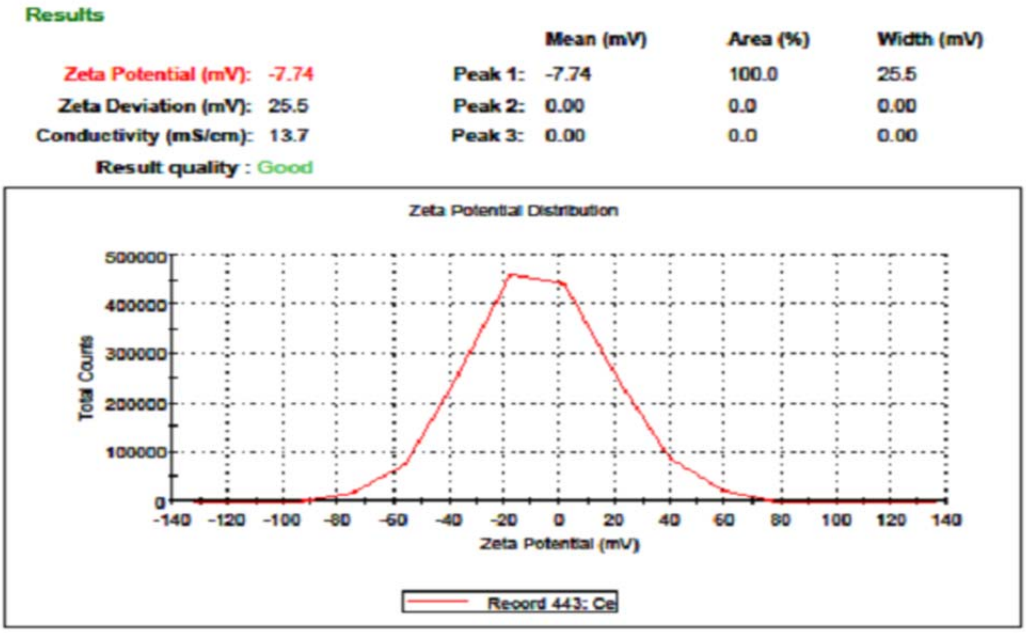


Fig. 6 - Electrophoretic mobility of CeO2 nanoparticles in DMEM

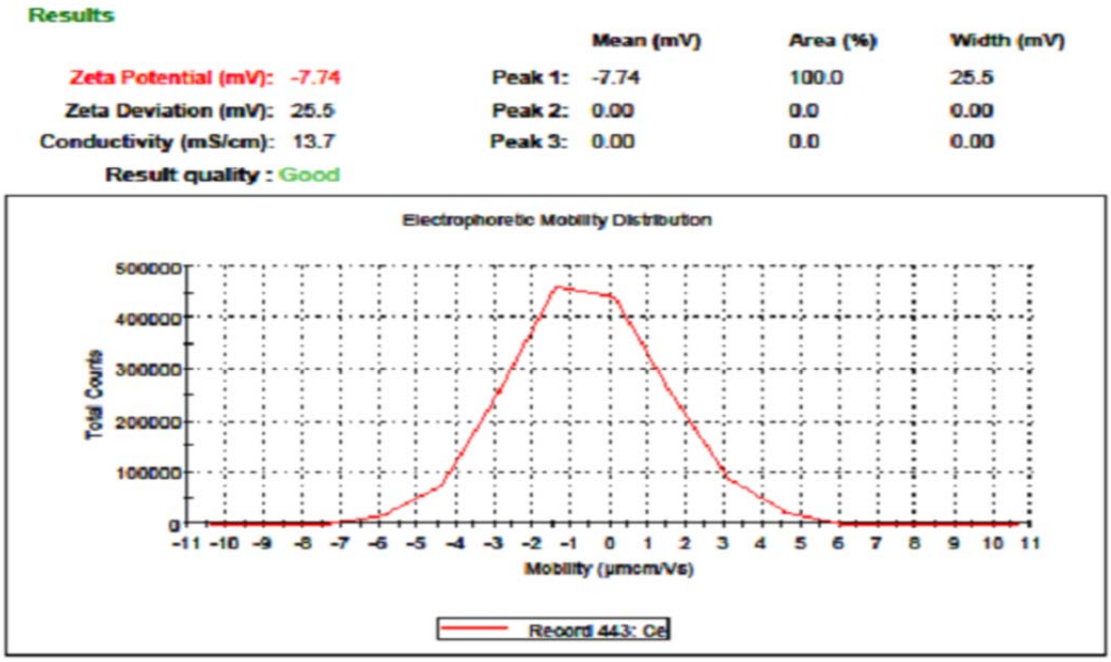


Fig. 7 - Size distribution of Co_3O_4 nanoparticles in DMEM

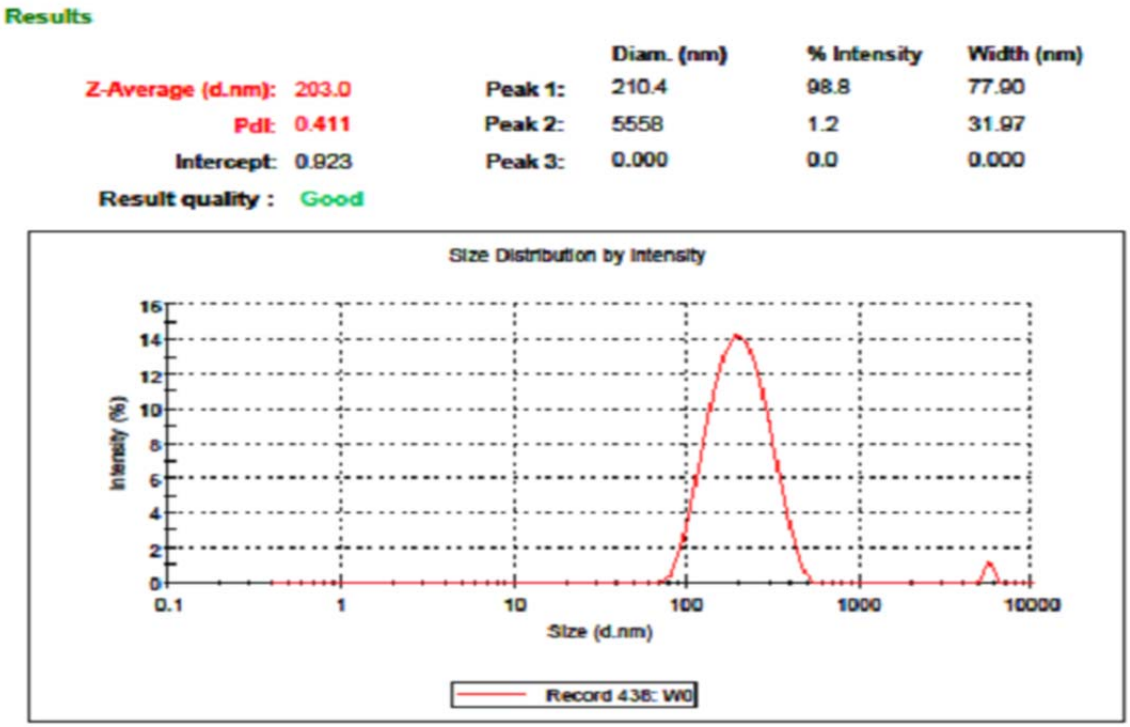


Fig. 8 - Zeta potential of Co_3O_4 nanoparticles in DMEM

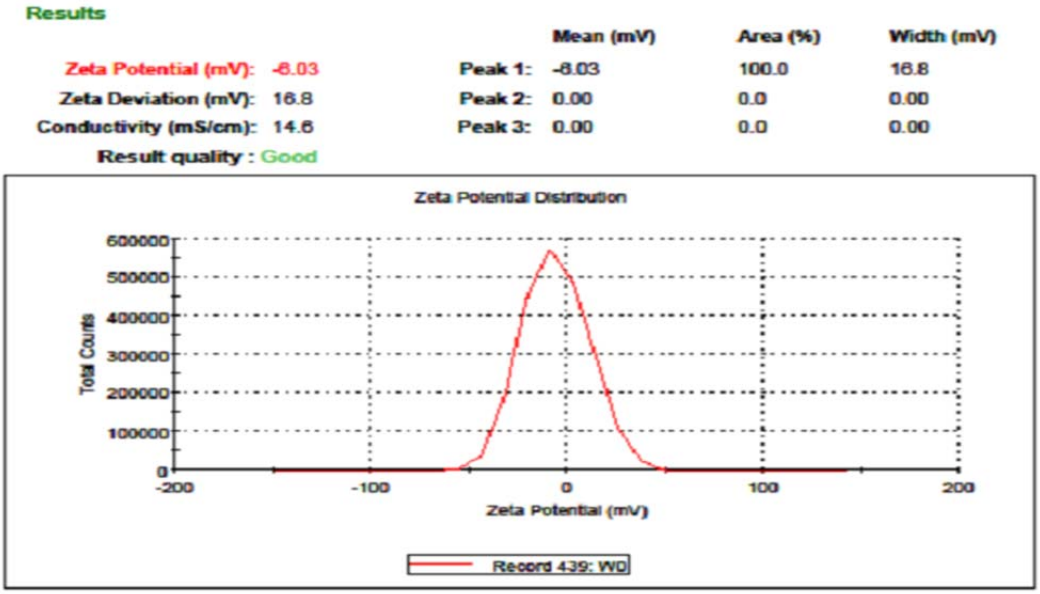


Fig. 9 - Electrophoretic mobility of Co_3O_4 nanoparticles in DMEM

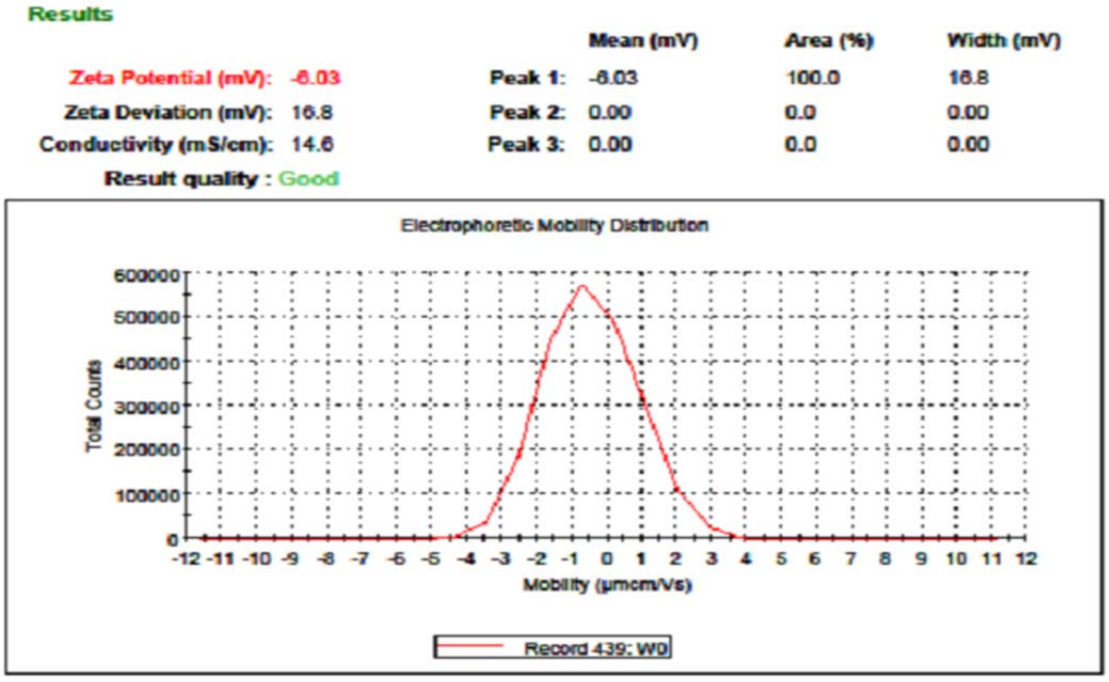


Fig. 10 - Size distribution of WO3 nanoparticles in DMEM

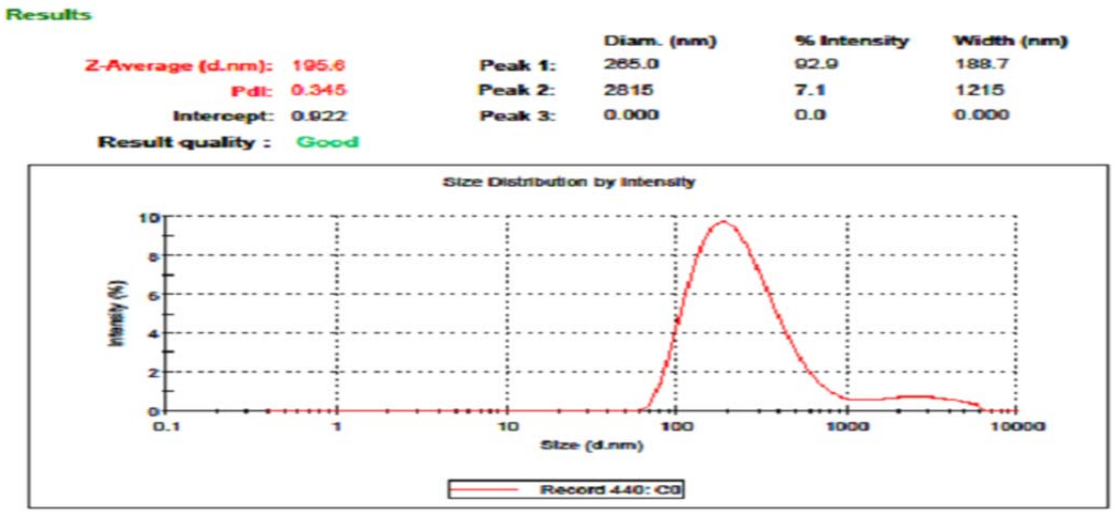


Fig. 11 – Zeta potential of WO3 nanoparticles in DMEM

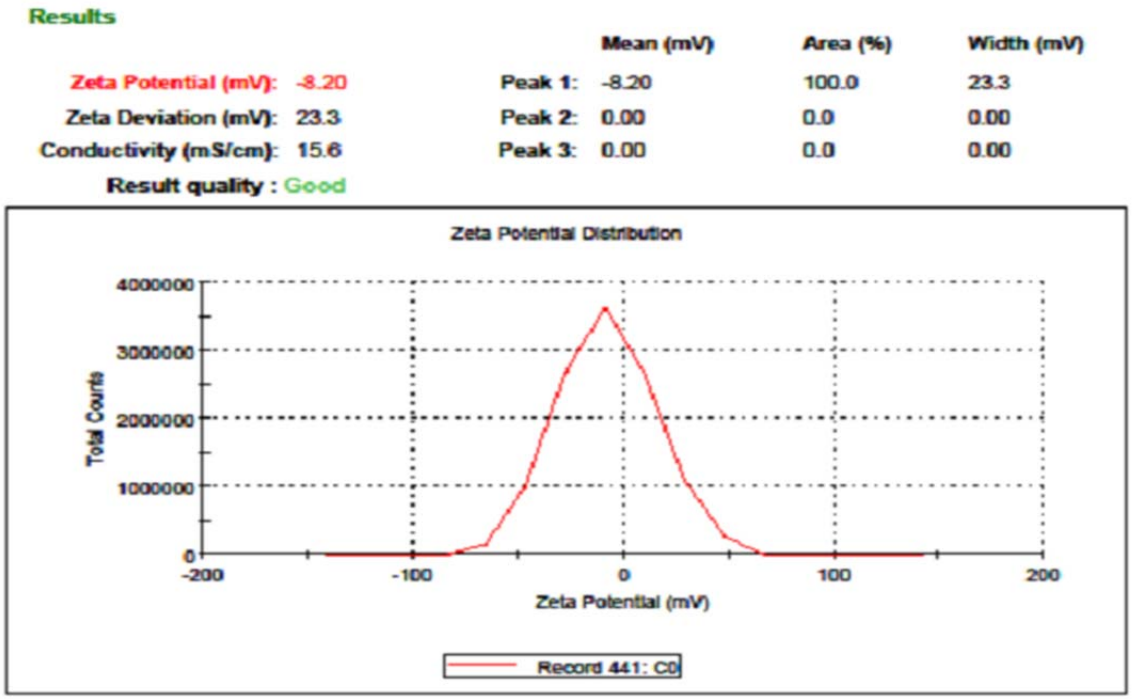


Fig. 12 - Electrophoretic mobility of WO3 nanoparticles in DMEM

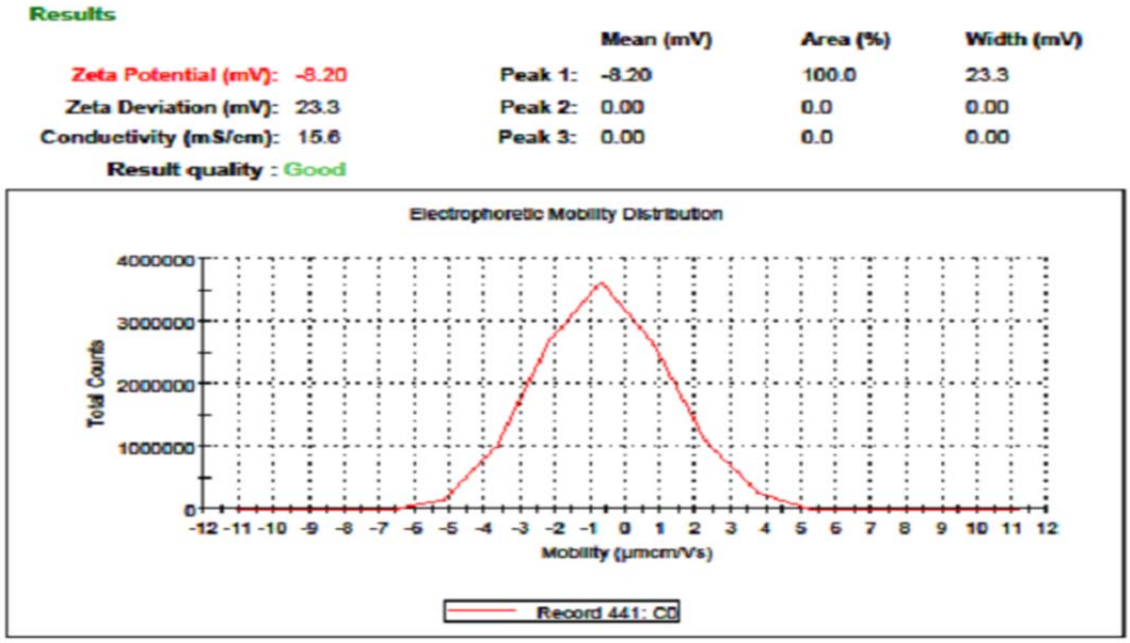
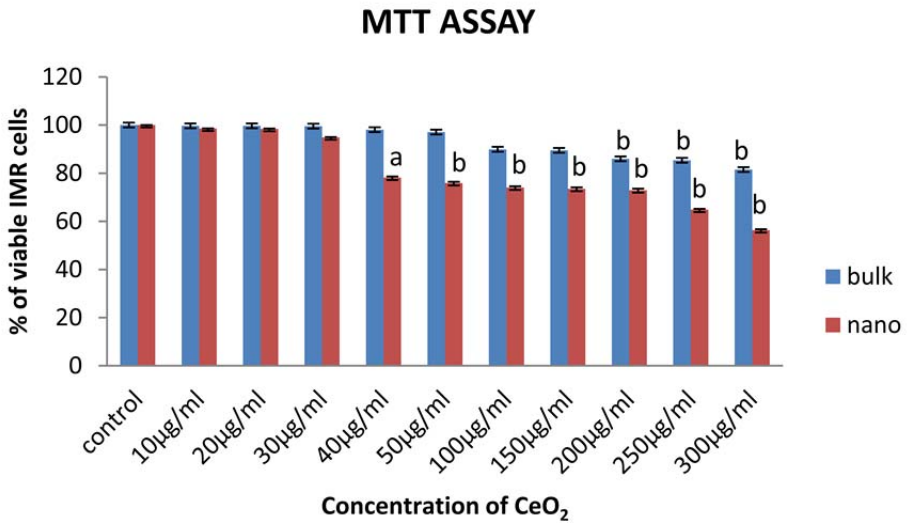
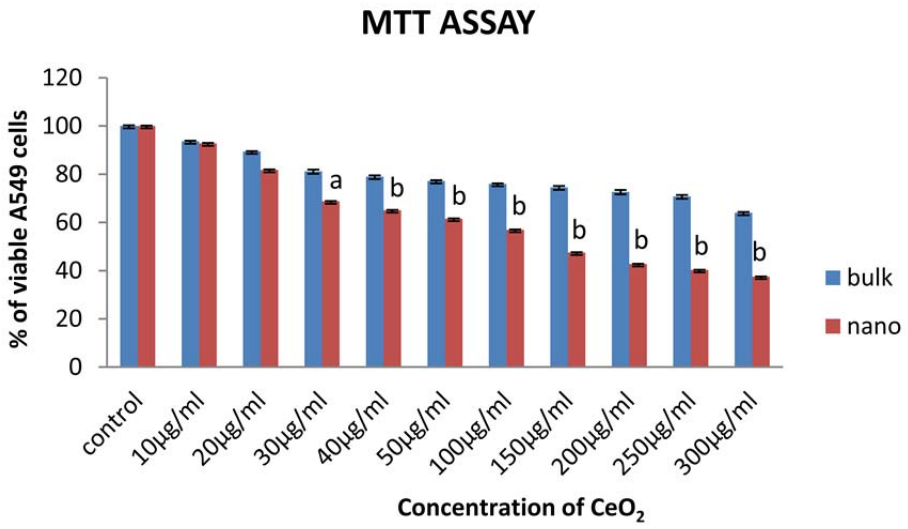


Fig. 13: Comparative effects of CeO_2 nano and bulk materials on viability of IMR 32 cell line



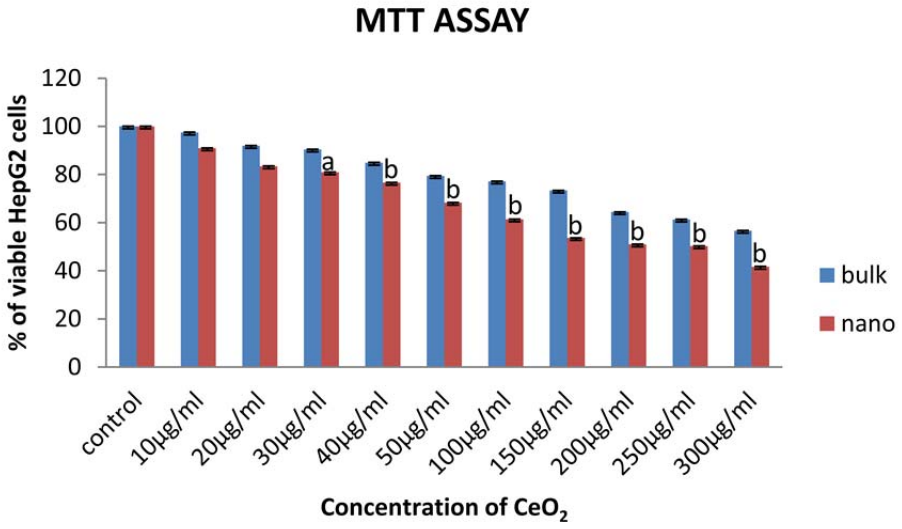
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Fig. 14: Comparative effects of CeO_2 nano and bulk materials on viability of A549 cell line



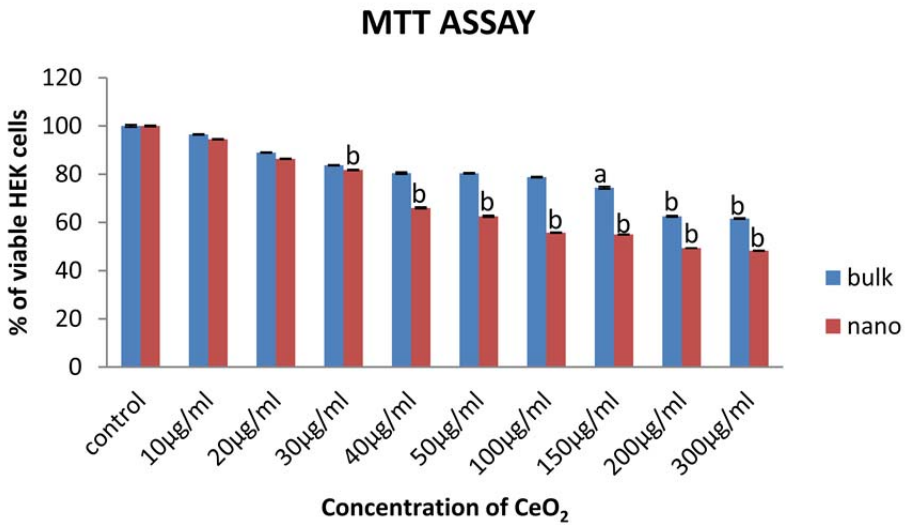
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Fig. 15: Comparative effects of CeO_2 nano and bulk materials on viability of Hep G2 cell line



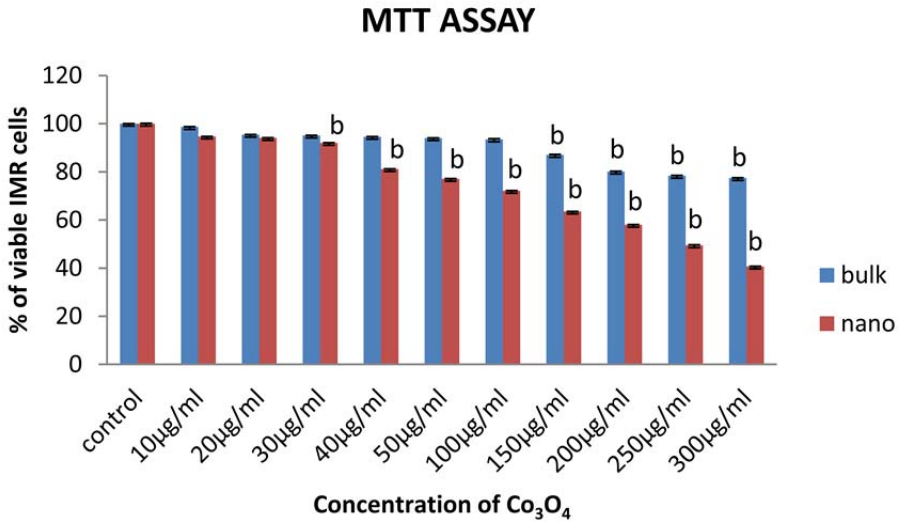
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 16: Comparative effects of CeO_2 nano and bulk on materials viability of HEK 293 cell line



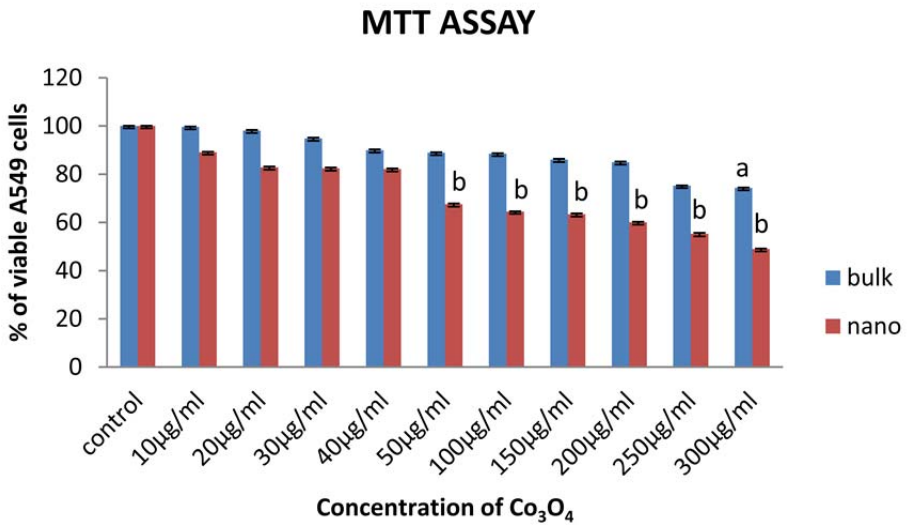
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 17: Comparative effects of Co_3O_4 nano and bulk on viability of IMR 32 cell line



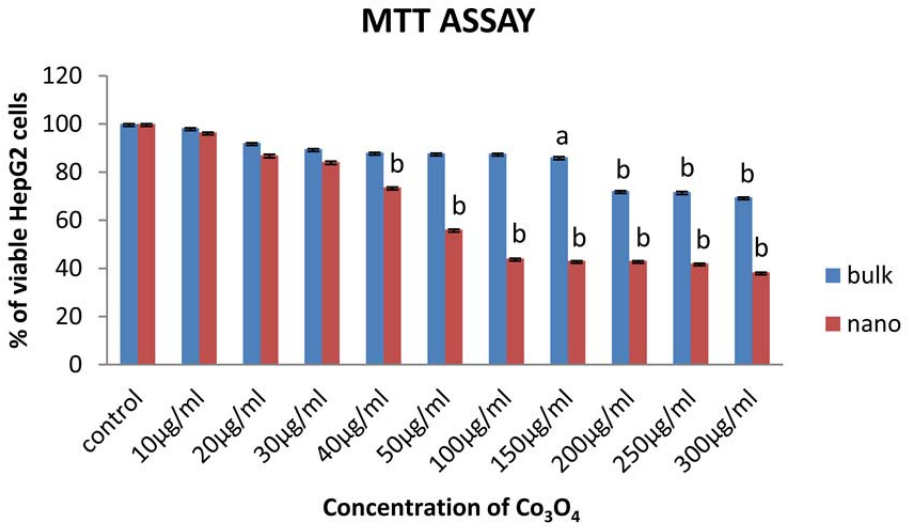
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 18: Comparative effects of Co_3O_4 nano and bulk materials on viability of A549 cell line



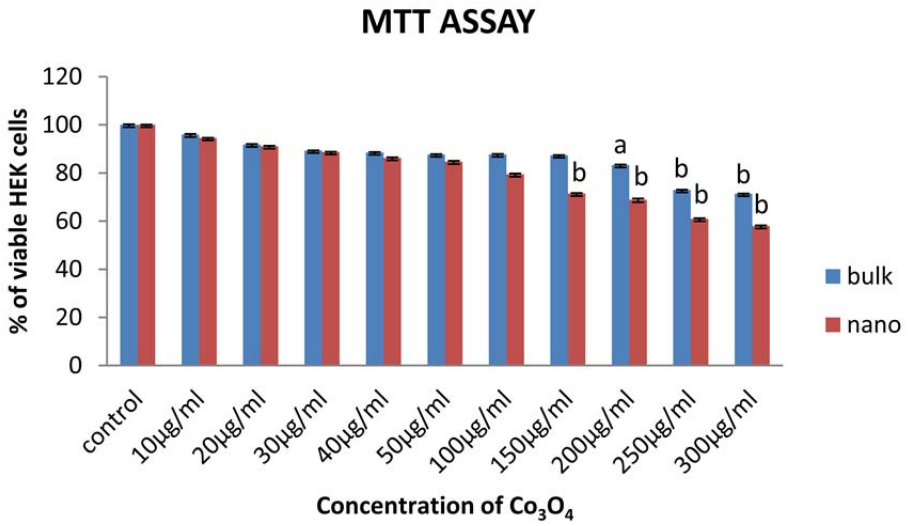
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Fig. 19: Comparative effects of Co_3O_4 nano and bulk materials on viability of Hep G2 cell line



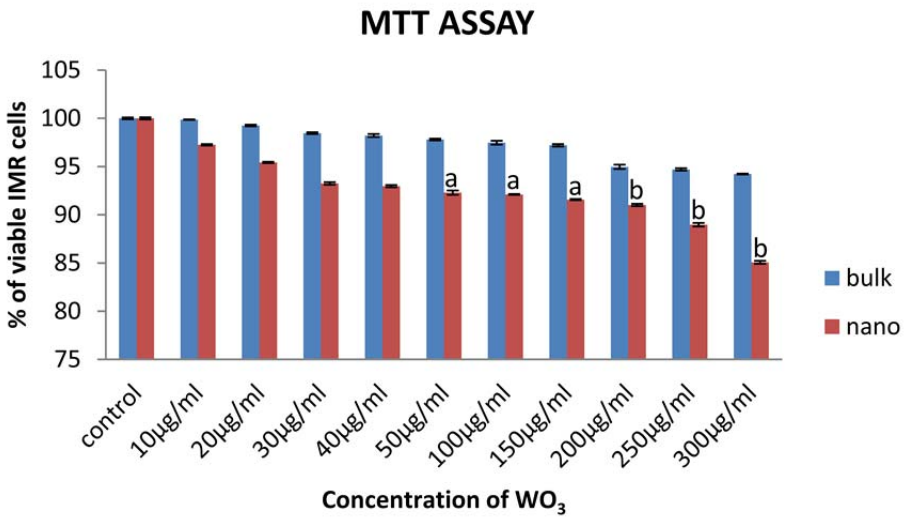
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 20: Comparative effects of Co_3O_4 nano and bulk materials on viability of HEK 293 cell line



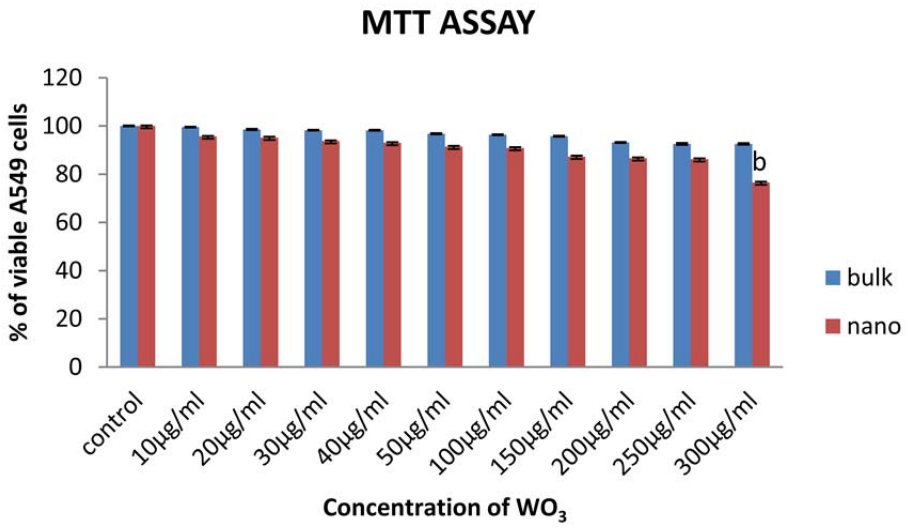
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 21: Comparative effects of WO_3 nano and bulk materials on viability of IMR32 cell line



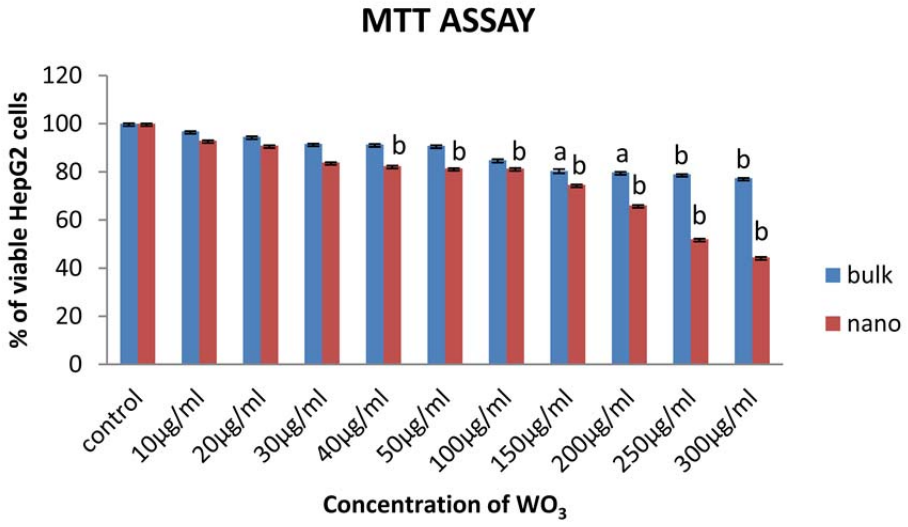
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 22: Comparative effects of WO_3 nano and bulk materials on viability of A549 cell line



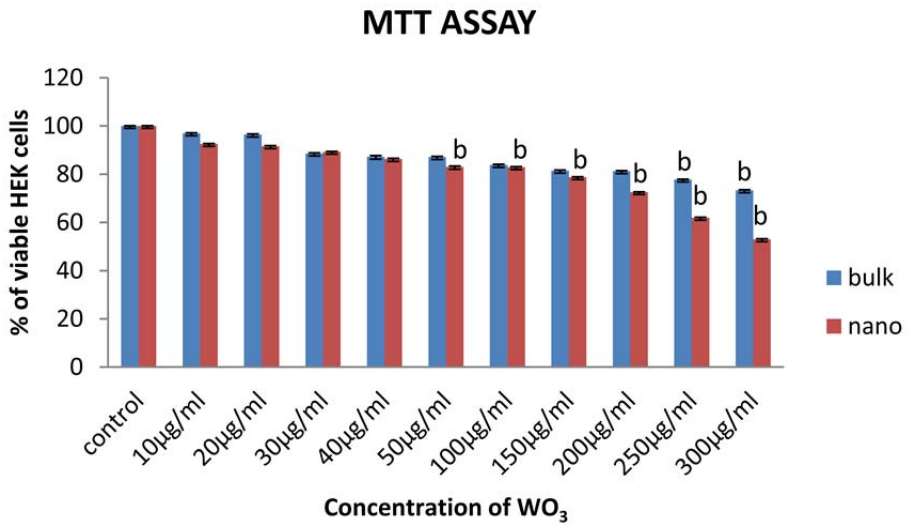
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 23: Comparative effects of WO_3 nano and bulk materials on viability of Hep G2 cell line



Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01

Fig. 24: Comparative effects of WO_3 nano and bulk materials on viability of HEK 293 cell line



Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Table 2: Showing IC_{50} of MTT assay by metal oxide Nanomaterials (CeO_2, Co_3O_4 and $WO_{3)}$ in different cell lines

Cell lines	CeO_2 (µg/ml)	Co_3O_4 (µg/ml)	WO_3 (µg/ml)
IMR 32	>300	248.94±89.98	>300
A549	180.80±53.71	>300	>300
HepG2	202.47±85.00	132.37 ±36.53	>300
HEK 293	180.91±80.80	>300	>300